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TRANSCRIPTIONAL REGULATION OF RIBOSOME BIOGENESIS IN SKELETAL MUSCLE GROWTH

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Transcriptional regulation or ribosome biogenesis in
skeletal muscle growth
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family,

ABSTRACT

Resistance exercise induces skeletal muscle hypertrophy via repeated exercise bouts. The increased muscle protein synthesis together with altered muscle protein degradation contribute to the adaptation of muscle mass. The number of ribosomes dictates the protein synthetic capacity of muscle. Therefore, the regulation of *de novo* ribosome synthesis is of great value for maintaining skeletal muscle mass and muscle hypertrophy. The main objective of this thesis is to examine the molecular mechanisms regulating ribosome biogenesis in response to different stimuli.

In **Paper I**, we investigated skeletal muscle gene expression following acute and chronic resistance exercise. By examining the phosphorylation of rpS6^{Ser235/236} as a marker for mTOR signaling pathway activated, we found that mTOR was activated after acute resistance exercise but attenuated after 12 weeks of resistance exercise. Similar to mTOR pathway, the 45S pre-rRNA and gene expression of c-Myc showed a dramatic increase after acute resistance exercise but reduced in response to acute resistance exercise following 12 week of resistance exercise. These findings suggest that the hypertrophy associated gene expression profile is not correlated with the developing muscle phenotype.

In **Paper II**, we exposed mice to 10% CO₂ and 21% O₂ in order to induce a hypercapnic environment and examined the responses of anabolic genes and proteolytic systems. Hypercapnia caused decreases in muscle fiber diameter, protein content and force production, which indicated muscle atrophy. Similarly, the increased level of CO₂ caused thinner myotubes, lower protein content and decreased anabolic signaling such as rDNA transcription rate. Meanwhile, the activity of ubiquitin-proteasome system was increased due to the increased AMPK-FOXO3-MuRF1 pathway. These results suggest that the coordination of protein synthesis and degradation is of great importance in the dynamic process of protein modification.

To further understand the regulation of muscle hypertrophy at the molecular level, we generated conditional skeletal muscle specific c-Myc knock out mice. In **Paper III**, we found that the mice lacking c-Myc displayed normal post-natal skeletal muscle development in terms of body weight, muscle weight, RNA content and rDNA transcription rate when compared to control mice. To challenge the c-Myc knock out mice in a rapid growth situation, we performed synergist ablation of the gastrocnemius and soleus muscles to induce compensatory hypertrophy of the plantar flexor muscles. The c-Myc conditional knock out mice showed normal hypertrophic response, which indicated that c-Myc is dispensable for hypertrophic growth in terminally differentiated cells. We further confirmed this finding by blocking c-Myc

function via chemical inhibitors in C₂C₁₂ myotubes. However, inhibiting c-Myc by siRNA or loss of its function by chemical inhibitor in proliferating C₂C₁₂ myoblast suggested a different role by blocking cell proliferation and decrease rDNA transcription. Therefore, we suggest a cell stage-specific role of c-Myc in the regulation of growth/proliferation and rDNA transcription in the myogenic cell lines.

To further understand the regulation of ribosome biogenesis during muscle hypertrophy, we focused on the mTOR pathway due to its regulatory role in protein homeostasis. In **Paper IV**, the mature C₂C₁₂ myotubes were stimulated with high concentration of serum to induce myotube hypertrophy. Increased mTOR signaling was detected during hypertrophy and the inhibition of mTOR prevented the hypertrophic response. When inhibiting the function of mTOR, rDNA transcription rate was decreased as well as the mTOR/rDNA promoter binding, which indicated a role for nuclear mTOR in the regulation of rDNA transcription. Furthermore, chemical inhibition of RNA Polymerases I prevented rRNA accumulation and myotubes hypertrophy, suggesting that intact Pol I mediated transcription was necessary for muscle hypertrophy.

In conclusion, this thesis investigates the regulation of skeletal muscle hypertrophy by different mechanisms. It highlights the importance of ribosome biogenesis during skeletal muscle hypertrophy.

LIST OF SCIENTIFIC PAPERS

- I. Nader GA, von Walden F, **Liu C**, Lindvall J, Gutmann L, Pistilli EE and Gordon PM. Resistance exercise training modulates acute gene expression during skeletal muscle hypertrophy. *Journal of Applied Physiology* (1985) 116, 693-702, 2014
- II. Jaitovich A, Angulo M, Lecuona E, Dada LA, Welch LC, Cheng Y, Gusarova G, Ceco E, **Liu C**, Shigemura M, Barreiro E, Patterson C, Nader GA, Sznajder JI. High CO₂ levels cause skeletal muscle atrophy via AMPK, FoxO3a and muscle-specific ring finger protein1 (MuRF1). *The Journal of Biological Chemistry*. 2015 Apr 3; 290(14):9183-94.
- III. **Liu C**, von Walden F, Guerrero-Cacais AO, Bruton J and Nader GA. c-Myc is dispensable for post-natal skeletal muscle development and work-induced hypertrophy. *Manuscript*
- IV. von Walden F, **Liu C**, Aurigemma N and Nader GA. mTOR regulates myotube hypertrophy by modulating protein synthesis, rDNA transcription and chromatin remodeling. *Manuscript*

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LIST OF ABBREVIATIONS

| | |
|-----------|---|
| 4E-BP1 | Eukaryotic translation initiation factor 4E binding protein 1 |
| AMPK | 5' adenosine monophosphate-activated protein kinase |
| AraC | Arabinofuranosyl Cytidine |
| bHLHZip | Basic helix-loop-helix leucine zipper |
| BrdU | 5-bromo-2'-deoxyuridine |
| BSA | Bovine serum albumin |
| cDNA | Complementary DNA |
| CFSE | Carboxyfluorescein diacetate succinimidyl ester |
| CKD | Chronic kidney disease |
| DAPI | 4,6-diamidino-2-phenylindole |
| DM | Differentiation medium |
| DMEM | Dulbecco's modified eagle medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DRB | 5,6-Dichloro-1- β -D-ribofuranosylbenzimidazole |
| eIF4A/E/G | Eukaryotic translation initiation factor 4A/E/G |
| ETS | External transcribed sequence |
| FACS | Fluorescence-activated cell sorting |
| FBS | Fetal bovine serum |
| FDB | Flexor digitorum brevis |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GM | Growth medium |
| GM+ | Growth medium with high serum |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HS | Horse serum |
| IGS | Intergenic spacer |
| ITS | Internal transcribed sequence |
| Mrf 2 | Myocyte regulatory factor 4 |
| mRNA | Messenger ribonucleic acid |
| mTOR | Mechanistic target of rapamycin |

| | |
|--------------|--|
| mTORC1 | mTOR complex 1 |
| mTORC2 | mTOR complex 2 |
| MuRF1 | Muscle RING-finger protein-1 |
| Myf5 | Myogenic factor 5 |
| NOR | Nucleolar organizing region |
| P70S6K1 | P70 S6 kinase 1 |
| PDCD4 | Programmed cell death protein 4 |
| PI3K | Phosphatidylinositol-4,5-bisphosphate 3-kinase |
| PIC | Pre-initiation complex |
| PIKK | Phosphoinositide(PI)3-kinase-related kinases |
| Pol I/II/III | RNA polymerase I/II/III |
| PVDF | Polyvinylidene difluoride |
| qRT-PCR | Quantitative real-time polymerase chain reaction |
| rDNA | Ribosomal deoxyribonucleic acid |
| rDNAp | rDNA promoter |
| RE | Resistance exercise |
| RIPA | Radioimmunoprecipitation assay buffer |
| RNA | Ribonucleic acid |
| RP | Ribosomal protein |
| rpm | Rotations per minute |
| rRNA | Ribosomal ribonucleic acid |
| siRNA | Small interfering RNA |
| SL-1 | Selectivity factor 1 |
| T2D | Type 2 diabetes mellitus |
| T+A | Training + Acute |
| TAF | TATA-binding protein associated factor |
| TBP | TATA-binding protein |
| TBS-T | Tris buffered saline-Tween 0.1% |
| TFIIIC | Transcription factor for polymerase III complex |
| tRNA | Transfer ribonucleic acid |
| TTF1 | Transcription termination factor I |

| | |
|-----|--------------------------|
| UBF | Upstream binding factor |
| UCE | Upstream control element |

1 INTRODUCTION

Skeletal muscle is the most abundant tissue in the human body, which transforms chemical energy into movement. Movement is essential for life in every aspect of health thus the normal physiology of skeletal muscle plays a fundamental role in health and disease. Under the conditions of neurological, infectious, metabolic, oncological, and many other disorders, loss of skeletal muscle mass (atrophy or wasting) significantly decreases quality of life and increases morbidity and mortality. Currently, the only efficient yet safe way to increase muscle mass in human is via resistance exercise (RE). RE training can increase muscle mass via repeated periods of augmented protein translation, resulting in protein accretion and muscle hypertrophy. Several evidences have been shown that in many disorders like Type 2 diabetes and chronic kidney disease (Ikizler 2011; Nader and Lundberg 2009; Lundberg and Nader 2008), RE can be an optional treatment besides the traditional medicine.

Therefore, the work described in this thesis focuses on the molecular mechanisms that controlling skeletal muscle mass, and more specifically the regulation of *de novo* synthesis of ribosomes. These findings may provide potential targets for the development of effective therapies and pharmacological agents aiming at preventing loss of skeletal muscle mass.

1.1 SKELETAL MUSCLE AND RESISTANCE EXERCISE

The skeletal muscle system represents one of the largest systems in the body and accounts for more than 40% of the body mass (Hoppeler and Fluck 2002). Skeletal muscle is composed of elongated cells, which are known as muscle fibers. Unlike other cell types, muscle fibers contain several nuclei, which are all located beneath the plasma membrane (sarcolemma). The satellite cells, which have only one nucleus, are located outside of the sarcolemma but within the basal lamina (basement membrane). Mature and functional muscle fibers are parallel to their neighbors and are arranged in bundles called fascicles and enclosed by connective tissue. Each muscle fiber is surrounded by a capillary network that supplies the cell with the necessary oxygen and nutrients required by the activated skeletal muscle. As the most abundant myofibrillar proteins, the contractile proteins myosin and actin occupy about 80% of the muscle fiber volume in human skeletal muscle (Eisenberg 2011). Due to this high protein content, the skeletal muscle represents as a reservoir of protein at the organismal level under the conditions of low nutrient availability (Finn and Dice 2006).

During skeletal muscle contractions, the glucose stored in the muscle is utilized by mitochondria. Thus skeletal muscle is also considered as primary insulin target as its

marked impact on glucose homeostasis (Richter and Hargreaves 2013). Meanwhile, many other events like phosphorylation of protein kinases or activation/deactivation of various groups of genes contribute to this adaptation to training (Bengt Saltin 1983). Thus a systematic profile on the expression patterns of genes may help people to understand the mechanism for the muscle adaptation to training.

The history of resistance training is often traced back to ancient Greece and the legend of Milo of Croton. Nowadays, the fact that RE training increases muscle mass and strength has become common knowledge and the beneficial effects on health in general are well described. Based on this knowledge, athletes can improve sport-specific performance in a more efficient approach and the general population may promote health, lean body mass and general fitness. Different mode, intensity, frequency and the volume of training can modulate acute resistance exercise. (American College of Sports 2009). The end-point results could also vary depending on the possible programs. Therefore, understanding the regulation of muscle under various conditions/time points is of great value for improving human health.

1.2 SKELETAL MUSCLE DEVELOPMENT AND HYPERTROPHIC GROWTH

From embryo to adulthood, the skeletal muscle mass are continuously increased. In skeletal muscle research, evidence of increased muscle mass can be either revealed in the form of increases in muscle size examined by magnetic resonance imaging (MRI), computer tomography (CT) or ultrasound (US). Alternatively, skeletal muscle mass can be indirectly evaluated by measuring protein content or distribution of cross-section area (CSA) assessed in muscle biopsy material. The general explanation for the increase in CSA was mainly due to the increases in fiber size and/or fiber number (hyperplasia) depending on different development stage (Macdougall 2003). During fetal development, the most important factor contributing to muscle growth is an increased fiber number. Several determination factors such as MyoD, Myogenic regulatory factor 5 (Myf5), Myogenin (MYOG) and the Myocyte enhancer factor-2(Mef2) are expressed in a time-dependent manner in the skeletal muscle progenitor cells (Buckingham et al. 2003). An increase in skeletal muscle fiber number is thus preceded by proliferation and differentiation of these progenitor cells. Both muscle mass and the number of myonuclei per fiber increased rapidly postpartum due to the accumulation of proteins and fusion of satellite cells (Layman, Hegarty, and Swan 1980). Skeletal muscle fiber number remains stable from roughly 3 weeks of age until adulthood (White et al. 2010).

In adult skeletal muscle, hypertrophy predominantly relies on an increased amount of contractile proteins, which leads to an increase in the size of preexisting muscle fibers (CSA) and their consequent force production (Macdougall 2003). However, evidence

in favor of an increased fiber number following chronic RE in humans has been presented (Larsson and Tesch 1986) and an increased number of myonuclei per fiber has been observed after mechanical overload (Bruusgaard et al. 2010). One potential explanation underlying both observations is that proliferation of satellite cells is responsible for these events (Allen, Roy, and Edgerton 1999). However, conditional deletion of satellite cells in skeletal muscle doesn't affect the ability of skeletal muscle to hypertrophy (Jackson et al. 2012; McCarthy et al. 2011). This result indicates that satellite cell proliferation and fusion with mature muscle fibers are not necessary for hypertrophic growth. Therefore, to understand the regulation of hypertrophic growth of preexisting skeletal muscle fibers, further studies on synthesis and accumulation of protein become essential.

1.3 PROTEIN HOMEOSTASIS IN SKELETAL MUSCLE

The protein homeostasis is of great importance for maintaining skeletal muscle function. It has been determined that as much as 280g of proteins are turned over per day in males weighing 70kg (Mitch and Goldberg 1996). Protein synthesis and protein degradation are dynamic processes that are simultaneously active, but in most of the situations they need to be coordinately regulated, depending on the circumstances, to balance the protein turnover. When overall rates of protein synthesis exceed the rate of protein degradation, the net protein turnover is positive resulting in accumulation of protein and skeletal muscle hypertrophy. Both protein synthesis (Chesley et al. 1992) and degradation (Phillips et al. 1997) are upregulated during acute resistance exercise (Kumar et al. 2009) or serum stimulation in cell (Zhang et al. 2014). Therefore a tightly regulated mechanism, which coordinates protein synthesis with protein degradation, is important to maintain amino acid and protein homeostasis, as any imbalance may disrupt cell or tissue function and/or morphology.

Protein synthesis begins with the transcription of DNA into mRNA, which then translocated and translated into amino acid chains by ribosomes followed by post-translational modifications and protein folding. Both translational efficiency and translational capacity are essential for the adaptation of protein synthesis rate. The rate of protein synthesis in skeletal muscle has been shown to be proportional to muscle RNA content (Millward et al. 1973). Considering that more than 80% of cellular RNA is made up of ribosomal RNA (rRNA), a sufficient amount of ribosomes are essential to guarantee efficient protein synthesis. Previous studies demonstrate that muscle RNA content increases by increased loading (Goldberg et al. 1975; Chaillou et al. 2013; Goodman et al. 2011) or following RE (Haddad, Baldwin, and Tesch 2005) and decreases by denervation (Haddad et al. 2003; Machida et al. 2012) or healthy aging

(Galavazi and Szirmai 1971). Therefore, the rRNA content dedicate to the protein translational capacity, which further contribute to protein homeostasis.

Protein degradation, on the other hand, is primarily believed to remove damaged and abnormal proteins and prevent their accumulation. Previous studies investigating the anabolic response of skeletal muscle to mechanical loading have often overlooked degradation in favor of synthesis measurements, as protein degradation has been considered to be more associated with pathological states than with normal cellular functioning. This line of reasoning was supported by the hypothesis that protein degradation is the principle mechanism underlying muscle atrophy and weakness (Bell, Al-Khalaf, and Megeney 2016). Removing of proteins, together with other function of protein degradation, such as sources of amino acids or regulation of cellular metabolism, are equally important in healthy muscle. In skeletal muscle, different proteins are degraded at different rates depending on the function and/or the relevant proteolytic pathway. Ubiquitin-proteasome (UP) mediated protein degradation targets short-lived proteins as well as autophagy-lysosome mediated protein degradation targeted long-lived proteins (Sandri 2013; Lilienbaum 2013). Dysfunction of the UPS and/or ubiquitin presenting proteins has been reported to reduce skeletal muscle atrophy (Caron et al. 2011) and induce muscle growth defects (Kitajima et al. 2014). Thus maintaining normal functions of protein degradation is essential for skeletal muscle.

Among many signaling networks, which participate in the regulation of protein homeostasis, the mTOR pathway is believed to represent a central signaling hub that synchronizes anabolic input from various sources and coordinately regulates both protein synthesis and degradation. RE has been shown to activate PI3K/mTOR/S6K (Al-Shanti and Stewart 2009; Glass 2010; Hulmi et al. 2009; Schiaffino et al. 2013) due to the multilevel regulatory functions of mTOR pathway. The role of mTOR will be further discussed in section 1.4.5.

1.4 RIBOSOME BIOGENESIS

Ribosomes are large ribonucleo-protein complexes measuring 200-250 Å in diameter and with an approximate mass of 4 MDa. Ribosomes are composed of two subunits with each of them containing rRNAs and ribosomal proteins (RP). In eukaryotes, the large subunit (60S) is composed of 28S rRNA (~4700 nucleotides), 5S rRNA (~120 nucleotides) and an additional 5.8S rRNA (~160 nucleotides) together with 49 ribosomal proteins. The small subunit (40S) is composed of 18S rRNA (~1900 nucleotides) and 33 proteins (Thomson, Ferreira-Cerca, and Hurt 2013). Ribosomal subunits are assembled in the nucleus and must be transported to the cytoplasm for their final maturation. The synthesis of protein from mRNA occurs on the ribosome in

the cytoplasm. The small unit (40S) binds to 5' end of mRNA then recognizes the correct tRNA, which charged with amino acid. Polypeptide chain elongates by the next tRNA binding to ribosome and catalyzed by the 60S subunit.

The amount of rRNA, as represented by total RNA content, has been reported increased following mechanical loading in animal models (Chaillou et al. 2013; Goodman et al. 2011; Miyazaki et al. 2011) or after resistance exercise in human (Haddad 2005). At the onset of skeletal muscle hypertrophy, rRNA content has been reported to increase approximately 2-fold (von Walden et al. 2012). It has also been shown that 24h to 48h serum stimulation in a rodent myogenic cell line (L6) results in a continuous increase in rRNA content (Nader, McLoughlin, and Esser 2005). On the contrary, inactivity, denervation and even healthy aging is associated with reduced rRNA content in skeletal muscle (Babij and Booth 1988; Galavazi and Szirmai 1971; Haddad, Baldwin, and Tesch 2005; Machida et al. 2012). Together these findings support an important role for ribosome content in influencing protein synthesis/accumulation and thereby the regulation of skeletal muscle mass.

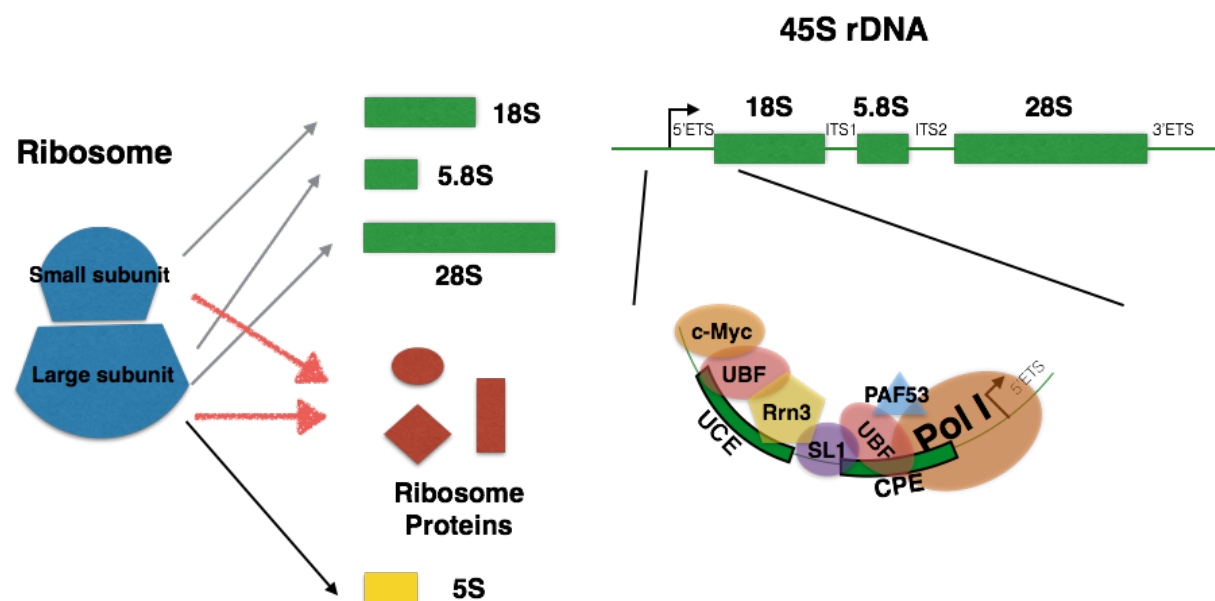


Fig. 1 Schematics demonstrating the components of the ribosome and the organization of the 45S rDNA gene.

1.4.1 Ribosome biogenesis

Ribosome biogenesis is a very energy consuming cellular activity and clearly a process of extraordinary complexity (Warner 1999; Fromont-Racine et al. 2003; Henras et al. 2008; Kressler, Hurt, and Bassler 2010). It requires the coordinated activity of all three nuclear RNA polymerases (RNA Pol I/II/III) present in eukaryotic cells. The rate-limiting step in ribosome biogenesis is considered to be RNA polymerase I (Pol I) dependent transcription, which is exclusively responsible for the transcription of the 45S precursor rRNA. This long precursor transcript is subsequently processed into the mature 18S, 5.8S and 28S rRNA species. RNA Pol I mediated transcription accounts for about 60% of overall transcriptional activity in the cell. RNA polymerase II (Pol II) transcribes all the mRNAs in the nucleus, including those coding for ribosomal proteins. RNA polymerase III (Pol III) is required for the transcription of 5S rRNA and tRNA. Counting together with all the rRNA and ribosomal proteins, more than 150 transacting factors and approximately one hundred small nucleolar RNAs (snoRNAs) involved in the maturation pathway of ribosomes (Kressler, Linder, and de La Cruz 1999; Venema and Tollervey 1999; Fatica and Tollervey 2002; Tschochner and Hurt 2003).

1.4.2 Polymerase I dependent 45S rDNA transcription

45S rDNA genes have about 400 copies per diploid genome in human and mice, and are located on 4-5 chromosomes (Moss et al. 2007). The nucleolus can organize around the rRNA genes during interphase, thus the regions where 45S genes are tandemly repeated were termed as nucleolar organizer region (NOR) or so called 45S rDNA sites (Lam, Trinkle-Mulcahy, and Lamond 2005; Shaw and Doonan 2005). It not clear that if NOR contribute to the regulation of cell proliferation but in non-proliferating cells it has been reported that the number of NORs is a marker of ribosomal protein synthesis activity in non-proliferating cells in skeletal muscle (Jozsa et al. 1993).

The length of the rDNA repeat is about 43 kb in human or 45 kb in mouse. The intergenic spacer (IGS) region is located between the transcribed regions and transcription termination signals are existed at both ends of IGS (Moss and Stefanovsky 1995; Hannan and Rothblum 1995). The function of the IGS is not entirely understood but it contains elements that can regulate or enhance the rDNA replication and transcription (Doelling, Gaudino, and Pikaard 1993). Based on the differences on function, the rDNA promoter can be divided into two distinct domains, the core promoter element (CPE) – with respect to the transcription initiation site, and the upstream control element (UCE) – essential for formation of the pre-initiation complex and promoting efficient rRNA transcription (Grozdanov, Georgiev, and

Karagyozyov 2003). Among different species, the sequence of the rDNA promoter does not show significant sequence identity. However there is a high level of conservation among species regarding the functional elements of the 45S rDNA gene (Paule and White 2000).

The transcription of rDNA begins with the promoter region and then continues with the external transcribed spacer (ETS), 18S rRNA, the internal transcribed spacer 1 (ITS1), 5.8S rRNA, ITS2 and 28S rRNA. The transcribed pieces generated from the rDNA varies from ~8 kb (yeast, *Drosophila* and *Xenopus*) to ~13 kb (mammals), partly contributed by the difference in the length of ETS and ITS (Moss and Stefanovsky 1995; Hannan and Rothblum 1995; Larson, Zahradka, and Sells 1991). At the onset of rDNA transcription, the pre-initiation complex (PIC), which is formed by several transcription factors, is recruited and assembled together with Pol I on the CPE. This protein complex is necessary for the initiation of 45S pre-rRNA synthesis. Within this multi-protein complex, the ribosomal DNA transcription factor Rrn3 (also referred to as Transcription Initiation Factor I (TIF-I) A), Selectivity Factor 1 (SL1), and Upstream Binding Factor (UBF) are essential for ribosome gene transcription in mammals. Once the transcription of 45S pre-rRNA precursor is complete, the transcript is processed into 18S, 5.8S and 28S following a number of events (Moss et al. 2007). There are several alternative processing pathways but a total of 4 sequences, two external and two internal, have to be excised in order to produce the mature rRNAs (Henras et al. 2015). The 5' external transcribed sequence (ETS) segments, which located at +650 bp on 45S pre-rRNA in mouse and +414 bp in human, is processed out first. (Kass, Craig, and Sollner-Webb 1987; Kent, Lapik, and Pestov 2009) Following its excision, the 5' ETS fragment is rapidly degraded and the half life of this primary cleavage product is estimated to be only a few minutes in both mouse and human (Popov et al. 2013). The fast clearance of the 5'ETS segment enables the approximation of Pol I transcription rate by quantification of this sequence, for example by Northern blot or qRT-PCR.

There are two potential targets for regulation of rRNA synthesis in response to the demand of cellular activities, the rate of Pol I transcription and the number of active rDNA genes. In general, not all the rDNA gene copies are continuously active (Conconi et al. 1989). French et al. has showed that a mutation yeast strain, which only have one third of the rRNA gene number but all active compared to the control, have similar grow rate as normal strain (French et al. 2003). They also suggested that this unaffected grow rate in mutation strain is likely due to the compensation by increased Pol I loading. This result suggested the important role for the regulation of rRNA synthesis by Pol I transcription in cell growth.

1.4.3 PIC factors and their functions

As mentioned above, the initiation of rDNA transcription requires assembly of the pre-initiation complex (PIC) at the rDNA promoter, which containing Pol I and a large number of auxiliary proteins. Each of these proteins is under control of various regulatory pathways, which collectively form a tightly regulated structure for the RNA Pol I mediated rDNA transcription.

TIFIA (Rrn3)

RNA polymerase I forms a stable complex with the transcription initiation factor Rrn3 through its subunit A43. (Milkereit and Tschochner 1998; Peyroche et al. 2000) Pol I subunit A49 also shows its contribution for the formation of the Pol I-Rrn3 complex and the subsequent dissociation. Functionally, TIFIA acts as a bridge for the other factors and facilitates their recruitment to the rDNA promoter. Once the transcription is initiated, TIFIA disassociates from the rDNA promoter region, unlike other factors, i.e. UBF, which remains bound (Milkereit and Tschochner 1998; Peyroche et al. 2000; Aprikian, Moorefield, and Reeder 2001; Bier, Fath, and Tschochner 2004; Beckouet et al. 2008).

UBF

In mammals, two Pol I-specific factors, the upstream binding factor (UBF) and the promoter selectivity factor (SL1), bind to the rDNA promoter in a synergistic manner, initiating the assembly of the PIC complex (Jantzen et al. 1990; Learned et al. 1986; Moss et al. 2007). UBF belongs to a family of proteins that contain high mobility group (HMG) boxes. The HMG boxes enable UBF to loop approximately 140 bp of DNA into a single turn, thereby inducing a nucleosome-like structure, called enhanceosome (Bazett-Jones et al. 1994). UBF can activate the transcription of rDNA directly by recruiting Pol I to the rDNA promoter or indirectly by stabilizing the binding of TIF-IB/SL1 or displacing nonspecific DNA binding proteins such as histone H1 (Kuhn and Grummt 1992; Kuhn, Stefanovsky, and Grummt 1993). Besides these functions, other roles of UBF has been suggested which including regulation of Pol I promoter escape (Panov et al. 2006) and transcription elongation (Stefanovsky et al. 2006). Moreover, UBF likely displays a more important role as compared to other PIC factors due to the reason that the UBF-rDNA association is not only limited in the promoter region but also extends through the entire transcribed region. This may suggest that UBF binding could contribute open chromatin state of active rDNA (Chen, Belmont, and Huang 2004). Depletion of UBF leads to reversible methylation-independent silencing of rRNA gene and reduction in the number of actively transcribed rDNA repeats (Sanij et al. 2008). Both elevated UBF mRNA and

protein levels and increased UBF-rDNA binding have been reported during skeletal muscle hypertrophy, together with an increased rDNA transcription rate (von Walden et al. 2012). Therefore the role of mTOR as a modifier for the relative proportion of active and silent rRNA genes together with its role as part of PIC indicated the critical position of UBF in regulation of Pol I mediated transcription.

The promoter of UBF gene contains an E-box motif (CACGTG), which can be recognized by the oncogene c-Myc and its heterodimers binding factor Max. c-Myc has been shown to regulate the UBF gene expression and hence indirectly enhance ribosome synthesis (Poortinga et al. 2004; Schlosser et al. 2003; Kim et al. 2000; Boon et al. 2001). We will further discuss the roles of c-Myc in section 1.4.4.

SL1

Pol I mediated transcription requires the TIF-IB/SL1 complex, a protein complex containing the TATA binding protein (TBP) and five TBP-associated factors, including TAFI110/95, TAFI68, TAFI48, TAFI35, and TAFI12 in mice (Zomerdijk et al. 1994; Heix et al. 1997; Gorski et al. 2007; Denissov et al. 2007). TAFI proteins undertake important functions in assembling of transcription complex, mediating specific interactions between the rDNA promoter and Pol I, and recruiting PIC together with Pol I to rDNA.

PAF53

Hanada et al. first reported the function of PAF53 in rRNA synthesis due to the correlation between the nucleolar accumulation of PAF53 and rDNA transcription. PAF53 is required for the initiation of rDNA transcription but likely not contributed to template binding elongation (Hanada et al. 1996).

All these reports suggest a mechanism whereby RNA Pol I and PIC complex are recruited to the rDNA promoter together, which acts as a scaffold to coordinate rDNA transcription.

1.4.4 c-Myc in regulation of ribosome biogenesis

1.4.4.1 c-Myc

One of the most studied groups of genes is the Myc oncogene family, comprising c-Myc, N-Myc, L-Myc, B-Myc and S-Myc. Among all the family members, most of the functional studies have focused on the c-Myc. The human c-Myc was discovered in the beginning of the 1980s and the protein was originally discovered as the homolog v-gag-myc, present in myelocytomatosis virus (Lee and Reddy 1999; Meyer and Penn 2008). Proper regulation of c-Myc expression is crucial for obtaining normal cell

functions. As it regulates the transcription of a wide range of genes, even small changes may influence cell growth, proliferation, apoptosis, differentiation and transformation (Meyer and Penn 2008; Levens 2010).

The C-terminal part of c-Myc contains a basic helix-loop-helix-leucine zipper (bHLH-LZ) motif, which interacts with the bHLHLZ motif of Max, forms a c-Myc/Max heterodimer. N-terminal to the HLH-LZ motif is the basic region (a.a. 355-369), which is involved in the c-Myc/Max binding to DNA but also necessary for full transformation of primary immortal cells (Meyer and Penn 2008). The c-Myc/Max heterodimer binds to specific DNA sequences (5'-CACGTG-3') named enhancer boxes (E-box) (Blackwood et al. 1991; Nair and Burley 2003). Heterodimerization with Max is necessary for c-Myc DNA binding and cMyc is not able to form homodimers (Lavigne et al. 1998).

1.4.4.2 Biological activities of c-Myc

As a transcriptional factor, c-Myc can regulate a wide range of biological activities including but not limited to cell proliferation, cell growth, differentiation, transformation and apoptosis etc. Based on the global transcriptional analysis, approximately 15% of the genes in the human genome are under transcriptional regulation of c-Myc (Orian et al. 2003; Li et al. 2003). For example, c-Myc directly activates the transcription of Cyclin D and Cyclin E genes, which are important for cell cycle regulation (Mateyak, Obaya, and Sedivy 1999). Other genes involved in regulation of cell metabolism (LDHA) (Shim et al. 1997), apoptosis (Bcl-2) (Bissonnette et al. 1992) and miRNAs (Tao, Zhao, and Tao 2014) are also considered to be the direct targets of c-Myc. On the other hand, c-Myc lies downstream of many key-signaling pathways in cells. Wnt/ β -catenin signaling can upregulate c-Myc expression which promote the cell proliferation (Zhang et al. 2012). JNK and mTOR have also been shown to regulate c-Myc through the phosphorylation at Ser62. (Noguchi et al. 1999; Cianfanelli et al. 2015). Therefore, c-Myc acts as a critical role by integrating environmental signals to a diverse group of cellular processes in proliferating cells.

1.4.4.3 c-Myc in ribosome biogenesis

The regulation of ribosome biogenesis by c-Myc has been reported by several evidence, especially on the regulation of Pol I transcription (Arabi et al. 2005; Grandori et al. 2005; Grewal et al. 2005). Considering that c-Myc is able to directly regulate the expression of UBF, together with the fact that siRNA silence of UBF prevented c-Myc from fully activating rDNA transcription, it suggests that c-Myc can indirectly regulate rDNA transcription via UBF (Poortinga et al. 2004). It has also

been shown that the presence of c-Myc occurred together with the protein-protein interaction with SL-1 and increased acetylation of histones. Furthermore, c-Myc also binds to the promoter and downstream rDNA regions close to the transcription termination site, which indicates a potential direct regulatory role of c-Myc via gene looping in rapidly growing cells (Keppel 1986). In the presence of c-Myc, serum stimulation rapidly leads to the formation of gene loop structures of rDNA, which may enhance the transcription rate (Shiue, Berkson, and Wright 2009). The regulation of Pol II and III dependent gene transcription has also been suggested in mammalian cells by c-Myc (Poortinga et al. 2011) and in *Drosophila* by the c-Myc homologue d-Myc (Gomez-Roman et al. 2003; Grewal et al. 2005). Together, these findings suggested a crucial role of c-Myc in the regulation of efficient cell proliferation and ribosome biogenesis in single cells (Oskarsson and Trumpp 2005; van Riggelen, Yetil, and Felsner 2010).

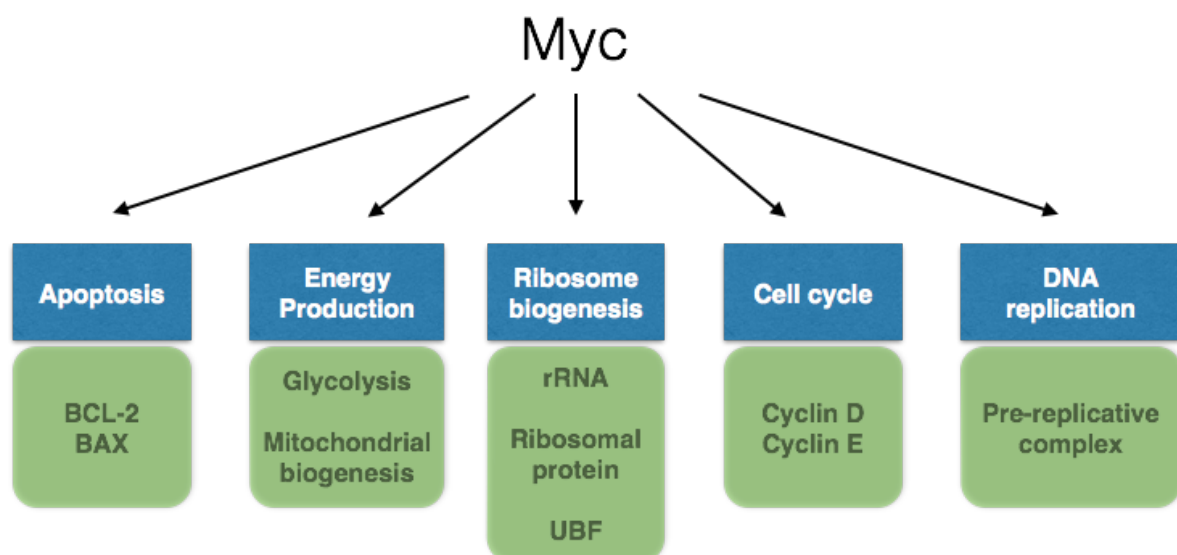


Fig. 2 The main cellular processes regulated by Myc together with some of the Myc regulated gene product.

1.4.4.4 *c-Myc in post mitotic cells*

A vast majority of the studies of c-Myc were conducted in a wide range of mitotic cells, i.e. different cancer-derived cell lines. It has been shown that c-Myc genomic deletion is lethal at embryonic day 10.5 (Davis et al. 1993). During embryogenesis, hematopoiesis has been suggested to be the crucial process, which interrupted by the absence of c-Myc and resulting in termination of embryo (Trumpp et al. 2001). Inhibition of c-Myc function severely impairs the growth-factor-induced proliferation

of cells in culture (Roussel et al. 1996). These growth deficiencies may not only be due to the crucial role of c-Myc in different cellular processes but also contributed by the connection between various cellular processes in eukaryotic cells. Failure or dysfunction of one key cellular activity (i.e. proliferation) will likely compromise other processes (i.e. cell growth) and eventually causes apoptosis. Therefore, by studying the mitotic cells, it is difficult to evaluate if c-Myc has a direct effect on one of the processes (i.e. Cell growth) as its broad role in other cellular processes.

However, the level of c-Myc protein is generally low in post-mitotic cells (Armstrong and Esser 2005; Poortinga et al. 2011). c-Myc has been described to act a special role in the hepatocyte proliferation that occurs during liver regeneration (Fausto et al. 1986; Thompson et al. 1986). A reduction level of hepatic c-Myc does not affect normal postnatal liver growth and development. Furthermore, Sanders has shown that reducing c-Myc does not affect the restoration of liver mass during liver regeneration or the restoration of liver protein following fasting (Sanders et al. 2012). Together, these findings indicate that c-Myc may have a different regulatory function in post-mitotic tissue comparing to proliferating cells.

In skeletal muscle, increased localization of MyoD and Myogenin on the rDNA promoter has been reported to be correlated with the decreased c-Myc protein level as well as decreased rRNA synthesis rate during the lineage progression (Ali et al. 2008; Bowman 1987; Zahradka, Larson, and Sells 1991). c-Myc gene expression also decreases stepwise during post natal development (Veal and Jackson 1998; Whitelaw and Hesketh 1992). In skeletal muscle from adult mouse, both protein and mRNA level of c-Myc are barely detectable (von Walden et al. 2012). However, hypertrophic growth of skeletal muscle as a result of growth factor stimulation and increased loading re-induced c-Myc (Armstrong and Esser 2005; Endo and Nadal-Ginard 1986; Nakahara et al. 2003; Osbaldeston et al. 1993; von Walden et al. 2012). Furthermore, aged skeletal muscle has shown a decreased ability to re-induce c-Myc as compared to young muscle, which might be considered to be one of the reasons for anabolic resistance (Alway 1997). All these data suggested that the role of c-Myc in skeletal muscle might vary depending on the development stages.

1.4.4.5 c-Myc in skeletal muscle glucose uptake

Due to the physiological demands of the human body, blood glucose concentration must be regulated within a tight range (Abdul-Ghani, Tripathy, and DeFronzo 2006; Wasserman 2009). Glucose concentration might vary under different conditions in terms of feeding or exercise. Thus preventing substantial disturbance in blood glucose concentration becomes essential for human.

Skeletal muscle is considered as the primary site of postprandial glucose uptake (Katz et al. 1983; Ferrannini et al. 1985). The skeletal muscle glucose uptake includes three different processes. First, glucose need to be delivered to the muscle fiber, then transported across the plasma membrane and finally phosphorylated by hexokinase to irreversible utilized glucose inside the muscle fiber (Wasserman and Ayala 2005). Glucose transport is believed to be the rate-limiting step for skeletal muscle glucose uptake (Wasserman and Ayala 2005). A group of membrane proteins in charge of this process and in skeletal muscle the principle glucose transporter protein is GLUT4 (Garvey et al. 1998; Goodyear et al. 1996). Considering the fact that c-Myc has been reported to regulate the genes for regulation of glycolysis (Kim et al. 2007) and several glucose transporter protein isoforms i.e. GLUT1 (Osthus et al. 2000), its potential role in regulation of skeletal muscle metabolism is yet to be revealed.

1.4.5 mTOR in regulation of ribosome biogenesis

The mechanistic target of rapamycin is a serine/threonine protein kinase, which belongs to the phosphoinositide 3-kinase-related kinases (PIKK) and is encoded by the mTOR gene in human (Harris and Lawrence 2003). It was discovered several decades ago that mTOR regulates cell growth, cell proliferation, protein synthesis, autophagy and transcription. Dysregulation or dysfunction of mTOR has been implicated as a contributing factor to several diseases (Jiang and Liu 2008). For example, disruption of mTOR leads to embryonic lethality at embryonic day 5.5 in mice (Gangloff et al. 2004; Murakami et al. 2004). Depending on which binding partners (like Raptor or Rictor) does mTOR associates with, two distinct complexes can form: mTOR complex 1 (mTORC1) or mTOR complex 2 (mTORC2). The mTORC1 pathway is believed to control many major processes via integrating inputs from several intracellular and extracellular resources like growth factors, stress, energy status and amino acids. On the other hand, it has been reported that mTORC2 activation regulates cytoskeletal organization and cell survival (Laplane and Sabatini 2012), however, the detailed function and regulation of mTORC2 required is less understood.

1.4.5.1 The mTOR pathway

The mTOR pathway is highly involved in the cellular response to nutrients by regulating ribosome biogenesis in response to amino acid availability. Downstream of mTOR, increased mTOR activity stimulates mRNA translation of protein and increased RNA transcription.

mTORC1 phosphorylates the translational regulators eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) at several residues which promotes the

dissociation of eIF4E from 4E-BP1. This protein-protein disassembling consequently reduces the inhibitory effect of 4E-BP1 and leads to formation of eIF4F complex, which containing eIF4A/E/G. In the present of mTOR, eIF4F complex binds to specific structure of mRNA, therefore promotes mRNA translation. On the contrary, when mTOR level is low, 4E-BP1 is hypophosphorylated and competitively binding to eIF4E and block translation initiation. Another subunit of the eukaryotic translation initiation factor, eIF4B, is believed to be a phosphorylation target of S6K1. Furthermore, mTOR phosphorylates (Brunn et al. 1997; Burnett et al. 1998) and thereby activates (Isotani et al. 1999) S6K1 (Thr 389), which in turn phosphorylates ribosomal protein S6 (rpS6). Besides rpS6 and eIF4B, the active S6K1 can also phosphorylates eukaryotic elongation factor 2 (eEF2) and thus promotes the translational elongation (Wang and Proud 2006). Therefore, by phosphorylates 4E-BP1 and S6K1, mTOR can regulate protein synthesis in multiply levels.

It has also been shown that mTOR regulates transcription by all three RNA polymerases through in several ways (Mayer and Grummt 2006). For the regulation of RNA Pol I mediated rDNA transcription, one of the functions of mTOR is that it regulates the activation/phosphorylation of PIC factors. Pol I was first reported to be rapamycin sensitive (Mahajan 1994). Thereafter, inhibition of the mTOR pathway was shown to result in inactivation of TIFIA by decreasing phosphorylation at serine 44 (Ser44) and enhancing phosphorylation at serine 199 (Ser199) (Mayer et al. 2004), which suggests that balancing phosphorylations with opposite effects may play an important part of mTOR dependent regulation (Drygin, Rice, and Grummt 2010). mTOR was also shown to control the phosphorylation of the retinoblastoma protein RB to release UBF (Nader, McLoughlin, and Esser 2005). Furthermore, phosphorylation of UBF can be increased due to elevated mTOR signaling, thus promoting its interaction with SL1 (Hannan et al. 2003). The binding of the SL1 complex to the rDNA promoter is also affected by mTOR following IGF-1 stimulation (James and Zomerdijk 2004). On the other hand, by direct interaction with the promoter regions, mTOR regulates the 45S rDNA, 5S rDNA and tRNA in a serum dependent and rapamycin sensitive manner (Li et al. 2006; Tsang, Liu, and Zheng 2010; Wei, Tsang, and Zheng 2009). mTOR phosphorylates and inhibits Maf1, a Pol III repressor, thereby inducing Pol III dependent 5S rRNA and tRNA transcription (Kantidakis et al. 2010).

mTOR signaling is also involved in the post transcriptional regulation of pre-rRNA, generally termed as rRNA processing. Maturation of the 45S pre-rRNA requires several stages of splicing. It has been reported that mTOR regulate the processing of pre-RNA in yeast (Powers and Walter 1999) and in human (Iadevaia et al. 2012) cells, although the underlying mechanisms have not been fully understood.

1.4.5.2 mTOR and muscle growth

mTOR is of great importance in all cell types. In skeletal muscle, phosphorylation of S6K1 was correlated with the increased muscle mass after resistance exercise (Baar and Esser 1999; Terzis et al. 2008). mTOR signaling was examined to be a crucial regulator of skeletal muscle mass as its regulatory function on protein synthesis (Anthony et al. 2000; Anthony, Anthony, and Layman 1999). Mechanical loading is sufficient to induce mTOR signaling (Goodman et al. 2011) and muscle hypertrophy was prevented when mTOR function was inhibited using rapamycin (Bodine, Stitt, et al. 2001). The activation of mTOR within skeletal muscle cells is sufficient to induce muscle hypertrophy (Goodman et al. 2011). Moreover, mTOR signaling can be induced by resistance exercise despite the absence of nutrition supply (Eliasson et al. 2006; Apro et al. 2013; Dreyer et al. 2006). Due to these critical roles of mTOR pathway in skeletal muscle, this thesis further investigates the mechanism by which mTOR regulates ribosome biogenesis.

1.5 PROTEIN DEGRADATION IN SKELETAL MUSCLE HYPERTROPHY

In eukaryotic cells, there are two major pathways, the ubiquitin-proteasome system and autophagy-lysosome system, responsible for the degradation of most the cellular proteins. The ubiquitin-proteasome system (UPS) accounts for the degradation of 80-90% of total proteins, which includes many regulated, short-lived proteins as well as abnormal or denatured proteins. On the contrary, autophagy is primarily responsible for the degradation of most long-lived proteins, but also for most of cellular organelles i.e. mitochondria, ribosomes. Both of these protein degradation pathways have been described to be activate in skeletal muscle under catabolic conditions (Bechet et al. 2005; Tassa et al. 2003).

1.5.1 Ubiquitin-Proteasome system

To be recognized by the UPS, the target proteins need to be labeled with ubiquitin in advance. Ubiquitin (Ub), a 76 amino acids protein, can be assembled as an ubiquitin chain by multiple moieties of itself and then bind to the lysine residues on the target proteins as a degradation marker. In this process, Ub first needs to be activated by the action of a Ub activating enzyme (E1) and Ub conjugating enzyme (E2) then presented to the substrate protein, which is selectively targeted by the E3 ubiquitin ligases. More Ub will be added to establish a poly-ubiquitin structure. Afterwards, the poly-ubiquitinated proteins will be recognized by 19S proteasome complex then be cleaved by de-ubiquitinated enzymes (DUB) and eventually enter the 20S core for degradation. The final products, the constituting amino acids, will be recycled by cells as resource of protein synthesis. Ubiquitin could also be recycled and used for the next target protein.

1.5.1.1 UPS in atrophy

Muscle atrophy occurs as the consequence of imbalance between anabolic and catabolic processes, when the rate of protein breakdown exceeds protein synthesis. Under many clinical conditions and chronic diseases, the loss of muscle mass may lead to muscle weakness or even increased mortality. It has been demonstrated almost 50 years ago that an increase in protein degradation can contribute to the loss of muscle mass following denervation and glucocorticoid treatment (Goldberg 1969). After that, MAFbx/atrogen-1 and MuRF1, two muscle-specific Ub-ligases, were reported to be key regulators of muscle atrophy (Bodine, Latres, et al. 2001; Gomez-Roman et al. 2003; Gomes et al. 2001). Both MuRF1 and MAFbx are maintained at low expression levels in resting skeletal muscle and can be rapidly increased due to a variety of stressors thus representing an early response to muscle loss in various species (Bodine and Baehr 2014). MuRF1 has been proven to degrade the myofibrillar proteins (Clarke et al. 2007) whereas MAFbx can target MyoD, a myogenic regulatory factor, to regulate the atrophy process. Moreover, the function of MuRF1 is also believed to control protein synthesis as elevated levels of protein synthesis has been reported in the MuRF1 knock out mice under certain atrophy conditions (Baehr, Furlow, and Bodine 2011; Koyama et al. 2008).

Recently, researchers have suggested that the role of the UPS in muscle may also be important for both integrity and growth. When conditional knocked out one of the essential 26S proteasome protein in muscle, a significant deficit was shown in muscle growth and force generation (Kitajima et al. 2014). Resistance exercise has been shown to increase both protein synthesis and protein degradation in skeletal muscle (Rennie and Tipton 2000). The mRNA level of MuRF1 and MAFbx were also found increased within several hours following acute resistance exercise, which indicated an rapid increased ubiquitin proteasome-mediated proteolysis after resistance exercise (Murton, Constantin, and Greenhaff 2008). However, Baehr et al. (Baehr, Tunzi, and Bodine 2014) found that chronic loading of mouse skeletal muscle using the synergist ablation model induced skeletal muscle hypertrophy by increased protein synthesis and degradation through a MuRF1 independent UPS, which is in contrast to previous studies (Cunha et al. 2012). This controversial data suggested that the correlation between UPS and protein synthesis during hypertrophy has not been clearly understood, and the function(s) of the proteasome are more extensive than its previously suggested by its role in muscle atrophy.

1.5.1.2 Transcription regulation of MuRF1 and MAFbx

MuRF1 and MAFbx might be regulated by a similar sets of transcription factors due to the fact that they are transcriptionally up-regulated together under most atrophy-

inducing conditions. The class O-type forkhead transcription factors (FOXO) is believed as the most potential transcriptional factor in regulation of MuRF1 and MAFbx. FOXO transcription factors (FOXO1, FOXO3a) have elevated expression level in skeletal muscle during certain forms of atrophy (Cho et al. 2010; Furuyama et al. 2003). It has also been reported that the activated FOXO1 can directly bind to the MuRF1 and MAFbx promoters (Waddell et al. 2008). Interestingly, FOXO1-promoter interaction was shown to be necessary but not sufficient to increase MuRF1 and MAFbx gene expression in cultured myotubes (Stitt et al. 2004).

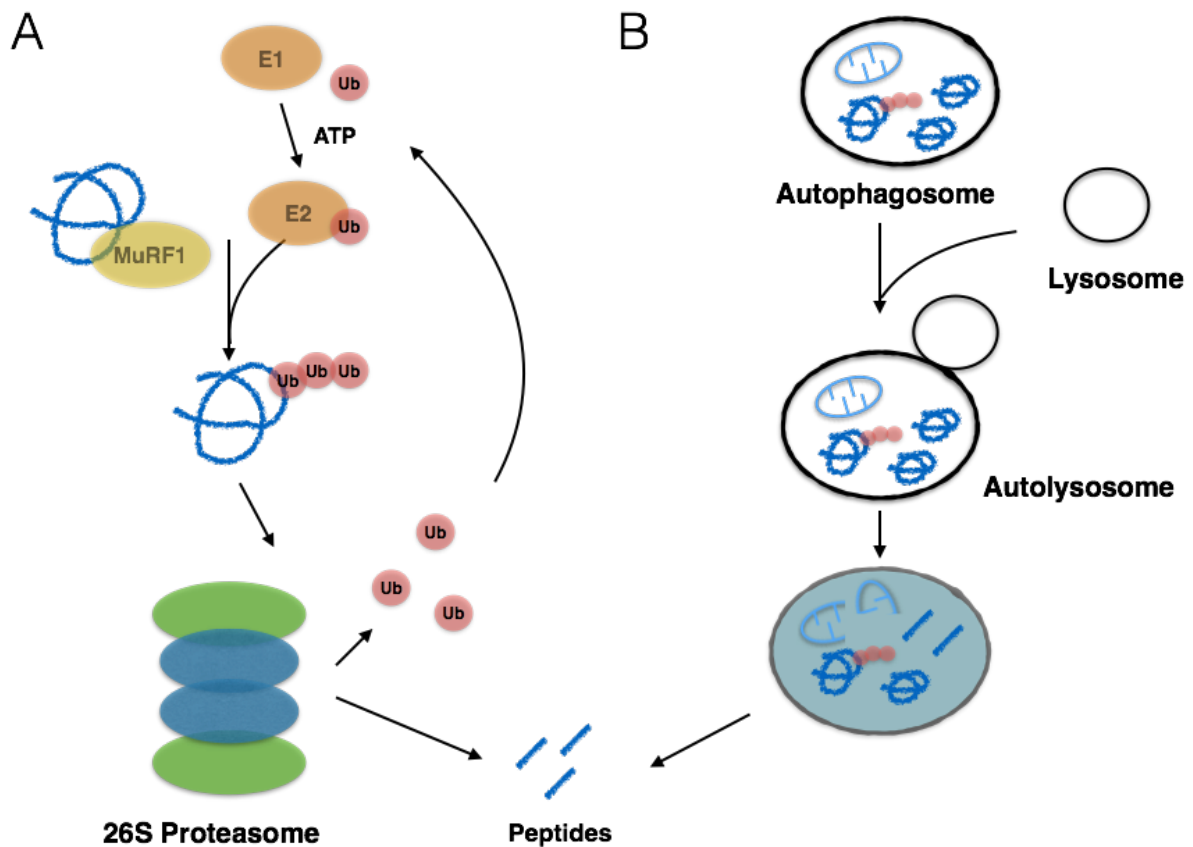


Fig. 3 Two major proteolytic pathways in skeletal muscle. A. Ubiquitin-proteasome system, B. Autophagy-lysosome system.

1.5.2 Autophagy

The autophagy-lysosome system degrades target proteins/organelles in a two-step process. The first step of autophagy is characterized ultra-structurally by the insolation of cytoplasmic constituents from the rest of cell into a double membrane-bound

vesicle called the autophagosome. Then the autophagosome subsequently fuses with the lysosome, leading to the degradation of its containing cytosolic proteins and organelles.

Autophagy was originally identified as a form of programmed cell death and often believed as one of the mechanisms that cause muscle wasting (Sandri 2013). In both mice and human, autophagy is activated by endurance exercise due to its potential role in removal of proteins and organelles that are damaged during exercise (Grumati et al. 2011). Autophagy has also shown a critical role in muscle adaptation and maintenance of healthy muscle.

2 AIMS

The main aim of the work presented in this thesis was to understand the mechanisms regulating ribosome biogenesis in skeletal muscle hypertrophy.

Objectives:

1. Elucidate the changing of genes expression before and after chronic training as a response to an acute bout of RE.
2. Understand the alterations in catabolic and anabolic signaling during hypercapnia-induced atrophy.
3. Determine whether c-Myc is necessary for skeletal muscle hypertrophy.
4. Investigate the coordination between RNA polymerases in response to growth stimulus in skeletal muscle cells.
5. Further investigate the function of mTOR in Pol I mediated transcription during muscle hypertrophy.

3 MATERIAL AND METHODS

3.1 CELL CULTURE

3.1.1 Culturing conditions for the C₂C₁₂ myogenic cell line

C₂C₁₂ myoblast were grown to 90-100% confluence in growth medium (DMEM with 10% FBS) and were induced to fuse into myotubes by being incubated in differentiation medium (DM: DMEM with 2% HS) for 4 days. To rid the terminally differentiated myotubes from undifferentiated myoblast, 20uM AraC was added to the medium for 24 hr on the third day after the induction of differentiation. Medium was changed every 2 days. At day 4 post differentiation, myotubes were either maintained in DM or stimulated with high serum medium (GM: DMEM with 20% FBS) for 1, 3, 6, 12, 48h. All experiments were performed in a humidified environment at 37 in a 5% CO₂ atmosphere. All the experiments were performed in triplicate.

3.1.2 Chemical treatment

Chemical inhibitors were used to block c-Myc function (Paper III) and PI3K, mTOR, p70S6K1, protein synthesis and Pol I and II (Paper IV). In Paper III c-Myc inhibition was accomplished using either Myra A (10-40 µM) (Mo and Henriksson 2006) or 10058-F4 (60- 100 µM) depending on the experiment. PI3K, mTOR and p70S6K1 inhibition was accomplished using LY294002 (20µM), rapamycin (25ng/ul) and PF-4708671 (20µM, Pearce et al 2010), respectively. Protein synthesis was inhibited by cycloheximide treatment (10-50µM) and Pol I and II by CX-5461 1µM (Drygin et al 2011) and DRB (75µM), respectively. DMSO was used as vector for all chemical inhibitors and the control group was supplemented with 0.1% DMSO. Medium was changed every day during stimulation.

3.1.3 siRNA induced gene silence

Paper III describes siRNA-mediated gene knockdown in C₂C₁₂ myoblasts. Cells were seeded in 6-well plates and transfected with c-Myc siRNA (50pM), Rrn3 (50pM) siRNA or control (50pM) siRNA. In Paper IV C₂C₁₂ myoblasts were seeded in 12-well plates and double transfected at Day 3 and Day 5 post differentiation with p70S6K1 siRNA (50pM), rpS6 siRNA (50pM) or control siRNA (50pM). All siRNA products used were purchased from Santa Cruz and Lipofectamine 2000 (Invitrogen) was used as a transfection agent according to the manufacturers instructions.

3.1.4 Assessment of cell proliferation

In Paper III, the effect of compromised c-Myc function on cell proliferation was assessed by two different approaches, 5-bromo-2'-deoxyuridine (BrdU)-incorporation assay detecting DNA synthesis and CFSE staining followed by fluorescence-activated cell sorting (FACS) to estimate number of cell divisions during a set period of time. For the BrdU-labeling assay myoblasts were grown on 8-chamber glass slides. After BrdU-incorporation, cells were washed with PBS followed by fixation in 15mM glycine-methanol solution for 20 min at - 20°C. Fixed cells were incubated with working solution for 30min, incubated with an anti- BrdU mouse antibody for 30 min and allowed to dry at room temperature, then covered with VectaShield Mounting Media containing 4,6-diamidino-2-phenylindole (DAPI). Visualization was performed using a multichannel Zeiss LSM-710 confocal microscope. For CFSE followed by FACS, myoblasts were labeled with 5μM CFSE for 10min at 37°C before plating and inhibitor treatment. After 48 or 72h samples were collected by trypsinization, labeled with Live/Dead cell dye (Invitrogen) for selection of viable cells only and run through a Gallios Flow Cytometer (Argon laser 488). Data analysis was performed using the Kaluza Analysis Software (Beckman Coulter, Brea, CA).

3.2 ANIMAL MODELS AND SURGICAL PROCEDURES

Generation of c-Myc^{D/D} mice, animal husbandry, surgical procedures and tissue collection: To generate a conditional skeletal muscle c-Myc deletion, we crossed c-Myc^{fl/fl} mice (8) with MCK-Cre^{+/-} mice (4). The F1 generation yielded Myc^{D/fl} mice, which were then intercrossed to obtain the F2 generation that yielded conditionally deleted c-Myc mice (c-Myc^{D/D}). F2 mice were born at the expected Mendelian frequency, displayed normal perinatal morphology, growth rates and fertility. Prior to any data collection and/or experimentation the F2 generation was backcrossed onto the C57Bl/6J c-Myc^{fl/fl} background for more than ten generations. Genotypes were verified in all mice by genomic PCR for MCK Cre, c-Myc floxed alleles and recombination at all breeding stages. Animals were kept on a 12:12-h light-dark cycle, had unlimited access to water, and were fed a standard rodent chow diet ad libitum.

3.2.1 Synergist ablation model

In Paper III a rapid and robust hypertrophic response was induced in the plantaris muscle by the synergist ablation model (Bodine and Baar 2012; Goldberg et al 1975). Functional overload was imposed by bilaterally removing soleii and gastrocnemii muscles. Control animals underwent a sham operation, where muscles were separated by blunt dissection, paying special attention to avoid any tissue damage. Animals were operated under surgical depth anesthesia, induced, and maintained with

Isoflurane (Baxter, Norfolk, UK). All procedures were approved by the local ethics committee and were carried out following Federation of Laboratory Animal Science Associations guidelines for animal experimentation (FELASA).

3.2.2 Glucose tolerance test

Mice were fasted for 6h by transferring mice to clean cages with no food or faeces in bottom of the cage but with the access to drinking water at all times. Body weights were measured and the volume of IP glucose injection (μl) was calculated as $10 \times$ body weight (g) as in 30% glucose solution. Baseline glucose level was measured once upon removed the tip of tail following by an intra-peritoneal injection with the appropriate amount of glucose solution. The blood glucose levels were further measured at 15, 30, 60 and 120 minutes by placing a small drop of blood on a new test strip and recording the measurement.

3.2.3 Single fiber preparation

To minimize the influence of non-muscle cells on assessment of degree of recombination, single muscle fibers were isolated and used for PCR (Paper III). Flexor digitorum brevis (FDB) muscles were cleaned of connective tissue, fat and blood vessels under a dissecting microscope using a pair of micro-iris scissors and jeweller's forceps. The clean FDB muscles were incubated for 2-3 hours at 37 °C in 0.3% Type I collagenase in DMEM supplemented with 10% FBS. Next, the muscles were transferred to fresh DMEM at 37 °C and gently triturated to produce a suspension of single muscle fibers. 300 μl of the resultant muscle fiber suspension was then placed in laminin coated glass-bottom Petri dishes and fibers were allowed to attach for 15 minutes before 2.7 ml DMEM supplemented with antibiotic, antimycotic solution (1 $\mu\text{L}/\text{ml}$) was added. Dishes containing muscle fibers were cultured for up to 48 hours at 37 °C.

3.2.4 Tissue collection

In Paper III mouse muscles were collected from live animals under surgical anesthesia induced and maintained by isoflurane. Excised muscles were cleaned of non-muscle tissue, blotted to remove blood and weighed on a precision scale (Sartorius Acculab ATL-84, Göttingen, Germany). Muscles were then snap frozen in liquid nitrogen, or mounted for histochemistry in OCT mounting medium and frozen in liquid nitrogen cooled isopentane. Following dissections, animals were euthanized by cervical dislocation.

In Paper I, muscle samples were collected bilaterally from the m. Biceps brachii using the Bergström needle biopsy technique (Bergstrom 1962). In order to facilitate sample

collection, suction was applied to the needle. Muscle biopsies were cleaned from visible fat and connective tissue, dry blotted and snap frozen in liquid nitrogen.

All samples were stored at -80°C until subsequent analysis.

3.3 HUMAN SUBJECTS AND RESISTANCE EXERCISE

In Paper I, 13 human subjects (six women and 7 men, mean age 24 ± 1.4 years) participated. None of the subjects had prior RE training history, were not diagnosed with a chronic disease and were dietary supplement- and medication-free. All subjects maintained their normal dietary habits during the study. All participants gave their informed written consent to participate, and the local ethical committee approved all procedures.

3.3.1 Group designation

Subjects were randomly allocated to one of two groups, the *training+acute* (T+A) or *acute* (A) group. Both groups trained the non-dominant (ND) arm for 12w and performed an acute bout of RE 7 days after the last training session, but differed with respect to treatment for the dominant (D) arm. T+A used the D arm as a non-exercise control and A performed the same acute bout of RE as described for the ND arm. Considering the aim of investigating acute gene expression before and after 12 weeks of RE training, we designated the D arms of those in the T+A group as *control* (i.e., no exercise), the D of the A group as *acute RE*, and the ND arm of both groups as *training+acute RE*.

3.3.2 RE training and dynamic strength testing

RE training consisted of a supervised, 12w progressive weight-lifting program of the upper arm 2 days per week as previously describe (Gordon et al. 2012). In short, upper arm exercises (biceps preacher curl, biceps concentration curl, standing biceps curl, overhead triceps extension, and triceps kickback) were performed for three sets using the six repetition maximum (RM) weight. Two min rest was allowed between sets. Prior to and after (48-72h after last training session) the 12w training program, dynamic strength was tested using the 1 RM weight for the elbow flexors (preacher curl). Two warm-up sets were completed at 50% and 75% of the predicted 1 RM for 8 repetitions and 5 repetitions, respectively. Single attempts were performed until one single repetition with full range of motion was completed. Dynamic strength gain was determined by calculating the percent difference between post- training and pre-training 1 RM strength.

3.3.3 Muscle CSA measurements

Magnetic resonance imaging (MRI) was used to observe changes in whole muscle cross-sectional area (CSA). Pre-training MRI scans were performed before or 48h after 1 RM testing and post-training MRIs were performed 48–96h after the last training session. To ensure precise and reliable measurements, six slices from each image were analyzed using bone morphological landmarks to ensure the same regions were measured pre- and post- training. Once the region of interest was segmented, total volume was determined for the six evaluated slices. Repeatability and reliability of Rapidia® volume measurements were verified using a phantom of known volume.

3.4 MATERIALS

C₂C₁₂ myoblasts were from American Type Culture Collection (Manassas, VA, USA). All chemicals were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA) unless otherwise stated. Horse serum (HS) was purchased from GIBCO (Grand Island, NY, USA). Laemmli buffer and DC Protein assay were obtained from Bio-Rad Laboratories (Hercules, CA, USA). TRIzol, Superscript VILO cDNA synthesis kit, Lipofectamine 2000, Carboxyfluorescein diacetate succinimidyl ester (CFSE) and Live/Dead cell dye were from Invitrogen (Invitrogen, Carlsbad, CA, USA). Complete Mini protease inhibitor cocktail and PhoStop phosphatase inhibitor were from Roche Diagnostics (Indianapolis, IN, USA). All siRNA oligos were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies used for Immunoblotting were from Santa Cruz Biotechnology, Cell Signaling (Beverly, MA, USA) or Abcam (Cambridge, UK).

3.5 PROTEIN ANALYSIS

In Paper I and III mouse and human muscle was homogenized in radioimmunoprecipitation assay (RIPA) buffer by use of a 5-mm generator coupled to a polytron (Kinematica, Kriens, Switzerland). In Paper IV cells were lysed in a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-based lysis buffer. The lysis buffer was supplemented with protease and phosphatase inhibitors. Following additional passive lysis at +4°C on a rotating wheel, lysates were spun down at 12,000 x g to pellet insoluble material. Supernatants were collected and protein concentrations assessed by the DC Protein Assay (Biorad, Hercules, California, USA). Protein homogenates were diluted with lysis buffer if needed and mixed 1:1 or 3:1 with 2X or 4X Laemmli buffer containing 5-10% β-mercaptoethanol. Samples were boiled at 95°C for 10 min and immediately cooled on ice and stored at -20°C until further use. Samples were separated by SDS-page on 7.5-12.5% polyacrylamide gels (Paper III) or precast Biorad gradient gels (4-20%) (Paper II and IV), depending on the size of the protein studied, and transferred to polyvinylidene difluoride (PVDF)

membranes activated in 100% methanol. Western blotting was performed using standard techniques: membranes were blocked in a protein-containing buffer, and washes were performed with Tris buffered saline- Tween 0.1% (TBS-T). Primary antibodies used were as follows: Paper I, rpS6 and PO4-rpS6 Ser235/236(1:1000, Cell Signaling) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:1000, Santa Cruz Biotechnology). Paper III, c-Myc (1: 1000, Abcam), c-Myc (1:500, Santa Cruz Biotechnology), RRN3 (1:1000, Abcam) and GAPDH (1:1000; Santa Cruz Biotechnology). Paper IV, mTOR, PO4-mTOR Ser2448, p70S6k1, PO4-P70S6k1 Thr389, rpS6, PO4-rpS6 Ser235/236, PDCD4 (1:1000, Cell signaling) and GAPDH (1:1000, Santa Cruz Biotechnology). All primary antibodies were diluted in a TBS-based buffer supplemented with 1% NaN₃ and 1% bovine serum albumin (BSA). Secondary antibodies (Anti-rabbit 1: 5000; GE Healthcare) were diluted in 5% milk in TBS-T.

3.6 PROTEIN SYNTHESIS MEASUREMENT

Myotubes grown in 6-well plates were maintained in DM, or stimulated with GM, GM + 25ng/ml Rapamycin or GM + 1μM CX-5461 for 24h after which cells were washed once with PBS and incubated in media containing ³⁵S- labeled methionine (40μCi) for 90min. Thereafter media was collected from each individual well as reference and cells were washed three times in cold PBS and lysed in cell lysis buffer. Lysates were spun down 5 min at 12000xg to pellet insoluble debris and supernatants transferred to new tubes. Lysates (15μl) were used to determine protein content using the DC protein assay (Biorad). Protein was then precipitated using TCA to quantify ³⁵S-incorporation. TCA precipitates were washed in Acetone-HCl (10%) and resuspended in elution buffer (100mM NaOH 1% SDS) at +55°C. ³⁵S incorporation was measured on a Wallac scintillation beta counter. Counts per minute (CPM) from protein samples were normalized to protein content (μg), incubation time and specific activity of the media for each well (CPM) using the following formula: Protein synthesis rate=((CPM (protein pellet)/protein in well (mg))/incubation time (h))/CPM (media).

3.7 HISTOCHEMISTRY

Myotubes were grown on the 4 well chamber slides. After stimulation with GM, cells were washed with Phosphate buffered saline (PBS) twice followed by 4% PFA for 20min. Then cells were permeabilized by PBS-T (0.1% Tween) for 10min, and washes were performed with PBS. Cells were blocked in 5% Goat serum in PBS-T. Primary antibodies were diluted (MHC, 1: 100) in blocking buffer and incubated with cells overnight. Secondary antibodies were diluted (1:200) in PBS. After three washes, VESTA SHIELD mounting Medium with DAPI was used for mounting.

Visualization was performed using a multichannel Zeiss LSM-710 confocal microscope.

3.8 RNA ANALYSIS

For total RNA extraction cells were collected in TRizol Reagent (Invitrogen) and subsequently extracted using Direct zol columns (Zymo Research). RNA purity and quantity were analyzed using a Nanodrop apparatus (xxx) and RNA integrity and column RNA size cutoff was verified by agarose gel electrophoresis. RNA was stored at -80°C until further use. cDNA was synthesized from 1µg of total RNA by the VILO cDNA synthesis kit accordingly to the manufacturer's recommendations. The cDNA stock was further diluted for downstream Quantitative Real-Time Polymerase chain reaction (qRT-PCR) depending on the target gene. qRT-PCR was performed using a SYBR-chemistry based supermix (GoTaq, Promega) on the BioRad CFX 384 system. Reactions were run in triplicate for each sample on 384 well, white hard-shell, clear well plates. Primers were tested on a 64-54°C heat gradient in order to optimize annealing temperature and verify a primer efficiency of approx. 100%. A melt-curve analysis was performed for each primer pair to ensure the amplification of a sole PCR product and amplicon size was verified by agarose-gel electrophoresis.

3.9 CHROMATIN IMMUNOPRECIPITATION

Chromatin Immunoprecipitation was performed on C₂C₁₂ myotubes and myoblasts (Paper III and IV) to assess protein association to promoter regions of interest. Sham and overloaded plantaris muscles were finely minced in a HEPES-based crosslinking buffer into a muscle slurry and cross-linked for 30 min in 1% formaldehyde. Myoblasts and myotubes were cross-linked by addition of formaldehyde directly to the cell culture media at a final concentration of 1%. The cross-linking reaction was quenched by the addition of glycine. Cells were collected in PBS and spun down at 1400 rotations per minute (rpm) for 5 min at +4°C. After removal of PBS, cells were lysed in ice- cold FA lysis buffer. Chromatin was sheered to produce DNA fragments of approximately 500-1500bp. Cross-linked and sonicated homogenates were stored at -80°C until further use. Sample DNA concentration was assessed using heat de-cross-linking overnight, proteinase K treatment and subsequent glycogen assisted precipitation of DNA following phenol/chloroform separation. The air-dried DNA pellet was diluted in RNase/DNase/nucleotide free H₂O and rehydrated for 1h at +65°C. DNA was quantified by Nanodrop at 260nm. 5-10µg of DNA was used per immunoprecipitation (IP) reaction. Samples were diluted to a final volume of 500µl. 2-3µg of antibody was added to each reaction and samples incubated overnight at +4°C on a rotating wheel. Magnetic beads were blocked over-night in a blocking buffer (glycogen, BSA and salmon sperm) and washed before diluted in IP dilution

buffer. 50µl of pre-blocked beads were added per reaction and samples incubated 60 min at room temperature. Beads were pulled down using a magnetic stand and washed several times. 150µl of elution buffer (1% SDS, 100mM NAHCO₃) was added to each tube and quickly spun down before heated at +30°C for 15 min. Eluted antibody-protein-DNA complexes were de-cross-linked, proteinase K digested and the DNA extracted as described above for input DNA concentrations. Samples were quantified using qPCR with primers specific for the rDNA promoter. Normalization was accomplished by normalization to rDNA signal of IgG control or to a non-related genomic region, respectively, and expressed as fold enrichment.

3.10 STATISTICAL ANALYSIS

Paper I

Differences in strength and CSA were compared using a two-way ANOVA with factors gender and training status followed by the Bonferroni post hoc test (significance level set at $P < 0.05$). Data are reported as mean \pm SE. Differences between groups for qRT-PCR data and Western blotting were determined using a one-way ANOVA, and significance between groups was established using the Newman-Keuls multiple comparison test ($P < 0.05$). All gene expression values are reported as means \pm SE.

Paper II

Data are expressed as mean \pm SEM. When comparisons were performed between two groups, significance was evaluated by the Student *t* test, and when more than two groups were compared, ANOVA was used followed by the Dunnett test, using GraphPad Prism software. Results were considered significant when $p < 0.05$.

Paper III

Data was analyzed by the Student T-test, one- or two-way ANOVA followed by Newman-Keuls or Bonferroni post hoc test depending on the experiment. The level of significance was set at $P < 0.05$ for all statistical comparisons. Values are reported as means \pm SD.

Paper IV

Differences between groups for total RNA, total protein, myotube diameter, gene expression and Western blot data were determined using the Student T-test or a one-way analysis of variance (ANOVA) followed by the Newman-Keuls post hoc test (if more than 2 groups were involved). Ch-IP data was analyzed independently per time point using one-way ANOVA followed by the Newman-Keuls post hoc test. The level

of significance was set at $P < 0.05$ for all statistical comparisons. Values are reported as mean \pm standard deviation (SD), unless stated otherwise.

4 RESULTS AND DISCUSSION

4.1 PAPER I

There are several reports showing that skeletal muscle adapts to chronic exercise due to a step-wise accumulation of different proteins and gain of function after repeated bouts. It has been widely studied on the gene expressions following the training. However, there is a lack of systematic profile on the expression patterns of a selected group of genes and little is known about the molecular mechanism underneath the RE training-induced adaptations. For example, genes involved in immune responses are attenuated following a short-term RE training as well as other genes responded in an opposite pattern (Gordon et al. 2012). One theoretically hypothesis is that following each exercise bout, an acute transcriptional response of higher magnitude occurs than that following the successive bouts. (Bickel et al. 2005). Thus this study investigates the acute response of four focused gene groups, which are all involved in skeletal muscle RE adaptation, to acute exercise in naïve and trained skeletal muscle.

13 untrained subjects underwent supervised RE training for 12 week of the non-dominant arm and performed an acute bout of RE 1 week after the last bout of the training program (Training + Acute). The dominant arm was either unexercised (control) or subjected to the same acute exercise bout as the trained arm (acute RE). By designing the subjects in this way, we have the chance to evaluate the differences between gene expressions in three different exercise stages – resting, acute exercise and acute exercise in the trained state (12 week training).

Twelve weeks of RE training resulted in morphological adaptations reflected as increased muscle CSA. Dynamic strength was also increased as an indication of muscle functional adaptation. Consistent with previous studies, an increased phosphorylation level of rpS6^{ser235/236} was observed after acute RE. However, following 12 week of RE training, the increased phosphorylation of rpS6 was attenuated. Compared to reports on altered mTOR signaling during by chronic resistance training (Ogasawara et al. 2013) and reduced protein synthesis in response to anabolic stimuli in trained skeletal muscle (Phillips et al. 1999), this reduction on rpS6 phosphorylation in training +acute to acute RE muscle may revealed the molecular mechanism behind RE training-induced adaptations.

We also focused on the ability of anabolic regulation after RE. It has been shown that the rate of protein synthesis rate is proportional to muscle RNA contents. Together with the fact that RNA Pol I mediated rDNA transcription is the rate limiting step for ribosome biogenesis, we found that pre-rRNA levels (ETS) increased two fold after acute RE. As one of the rDNA transcription regulators, c-Myc was also found a

remarkable increase after acute RE. This evidence supports the previous findings on ribosome biogenesis in response to acute mechanical loading across mammalian species. Consistence with decreased anabolic signaling i.e. attenuated phosphorylation of rpS6 in training+ acute group, both ETS and c-Myc were found reduced in response to acute RE following 12 week of RE training compared to acute RE. Furthermore, we also found that following 12 week of RE training, changes in gene expression are multidimensional, as not all the genes responded to acute RE and the responses could be either enhanced or suppresses following training state. Therefore, the correlation between the changing of gene expression profile and developing muscle phenotype is likely depending on the gene function.

In summary, this paper provides novel data indicating that changes in gene expressions following acute RE are multidimensional, and do not necessarily reflect the actual adaptive response that taking place during the training process.

4.2 PAPER II

Loss of skeletal muscle mass and/or muscle dysfunction is a common feature in aging and some diseases, e.g. chronic obstructive pulmonary disease (COPD). For the COPD patients, due to a disturbance in pulmonary gas exchange, both transportation of CO₂ from muscle and transportation of O₂ to muscle are impaired. It has been shown that high CO₂ levels can activate signaling pathways that impair the innate immune response or the functions of other organs (Wang et al. 2010; Helenius et al. 2009; Gates et al. 2013). However whether this CO₂ retention (hypercapnia) induces skeletal muscle atrophy is still yet to be answered. Thus this study investigated the activation of the proteolytic systems and suppression of the anabolic systems in hypercapnia induced skeletal muscle atrophy.

To investigate whether hypercapnia can causes skeletal muscle atrophy, mice were exposed to 10% CO₂ and 21% O₂ (hypercapnia) or to room air (normocapnia). The wet weight of the soleus muscle under hypercapnia was 10% less as compared under normocapnia. Both muscle cross-section area and strength were also found decreased in mice exposed to hypercapnia. As decreases in protein content, muscle fiber diameter and force production are the basic evaluation factors of skeletal muscle atrophy, our data in mice were similar to those observed in hypercapnic patients with COPD (Dick et al. 1997). To further confirm this hypercapnia induced atrophy phenotype, we addressed this phenomenon using the *in vitro* C₂C₁₂ myogenic cell line. Increased level of CO₂ caused thinner myotubes and lower protein contents in consistent with our *in vivo* results.

During muscle atrophy, proteolytic systems are activated and contribute to wasting of muscle fibers. To investigate the involvement of the ubiquitin proteasome system in the hypercapnia induced atrophy, proteasome inhibitor MG-132 and the ubiquitin-conjugating enzyme inhibitor UBE were applied to treat with mature myotubes. Inhibition of UPS prevented the hypercapnia-induced reduction in myotubes diameter. Considering that the specificity of the UPS is carried out by ubiquitin ligases E3, we further investigated the function of muscle-specific Muscle Ring Finger-1 (MuRF1) during hypercapnia. The protein and mRNA level of MuRF1 is upregulated following hypercapnia, and suppression of MuRF1 by siRNA had a protective effect against the hypercapnia induced atrophy. To further validate these findings, MuRF1 knock out mice were exposed to high CO₂ for 21 days. Consistent with our previous findings, absence of MuRF1 in muscle prevented the hypercapnia-induced reduction in both morphology and strength.

AMPK has been shown to mediate skeletal muscle protein degradation via atrogin-1 and MuRF1 (Nakashima and Yakabe 2007). Furthermore, acetyl-CoA carboxylase (ACC) phosphorylation of AMPK and nuclear FoxO3 showed increased patterns compared to MuRF1 when myotubes were treated with high CO₂. siRNA interference with different AMPK subunits or FoxO3 suggested that AMPKα2 and FoxO3 were necessary for CO₂ induced decrease in myotubes diameter and indicated that hypercapnia causes muscle atrophy via AMPK-FoxO3-MuRF1 signaling pathway.

In summary, hypercapnia-induced atrophy required increased catabolic signaling pathways such as AMPK-FoxO3-MuRF1 as well as decreased anabolic signaling exemplified by rDNA transcription. This finding indicated that in the dynamic process of protein modification, coordination of protein synthesis and degradation is of great importance.

4.3 PAPER III

The upregulation of c-Myc and 45S pre-rRNA induced by acute RE in skeletal muscle has been studied in Paper I. Among many functions of c-Myc, the regulation of ribosome biogenesis is a key factor for cellular growth control. While c-Myc is relatively well characterized in proliferating cells, little is known about the role of c-Myc in post-mitotic tissues, i.e. skeletal muscle. To understand the role of c-Myc in skeletal muscle and in transcriptional regulation of RNA Pol I/II/III, we started from the test of whether c-Myc is necessary for post-natal skeletal muscle development and hypertrophy following increased load.

Using the Cre-LoxP system, we generated a transgenic mouse model, in which c-Myc is specifically knocked out in mature skeletal muscle fiber. To avoid the effect of c-

Myc depletion during the developmental stage (Trumpp et al. 2001), we chose the muscle creatine kinase promoter (MCK) to drive Cre, because MCK is only expressed in mature skeletal muscle fibers. This mouse model helps us to investigate the effect of c-Myc in post-mitotic myofibers, instead of myogenesis.

From post-natal development throughout adult age (up to 48 week), no differences were detected between genotypes in terms of body weight, perinatal mortality or developmental defects. Skeletal muscle mass were examined by the wet weight of Plantaris, Tibialis anterior or Extensor digitorum longus and cross-section area of plantaris muscle. Surprisingly, no morphological changes were observed in c-Myc conditional knock out mice compared to control. Despite a significant reduction in c-Myc mRNA levels up to 12 week, the absence of c-Myc did not affect basal transcription of 45S rDNA by Pol I or 5S rDNA by Pol III. The gene expression level of several PIC factors, also known as c-Myc targeted genes, displayed a similar pattern compared to control. Although these results contradict our initial hypothesis that absence of c-Myc would prevent muscle growth by limiting foremost rDNA transcription and thereby ribosome production, these novel findings indicate that c-Myc is dispensable for rDNA transcription and post-natal skeletal muscle development.

To challenge the role of c-Myc during rapid growth following mechanical overload, we used a rodent model of skeletal muscle hypertrophy, synergist ablation of plantar flexor muscles (Goldberg et al. 1975). Gastrocnemius and Soleus were removed bilaterally resulting in increased mechanical loading of the remaining Plantaris. To avoid the influence of other tissue responses/activities i.e. inflammatory activity, other than the increased loading on skeletal muscle, we performed a bilateral sham operation on the control group animals, including skin and fascia incisions, manipulation of muscles and exposure of tendons but no removal of muscles (von Walden et al. 2012). Compared to the barely detectable expression level of c-Myc in adult skeletal muscle, c-Myc increased dramatically but much less of increase in the c-Myc conditional knock out mice. One of the potential explanations for the observation that c-Myc was detectable in c-Myc conditional knock out mice could be contribution by non-muscle cells. Skeletal muscle in lack of c-Myc displayed a similar response to mechanical overload when compared to control i.e. similar muscle mass increase, RNA content increase and more portions of large fibers. Thus, work-induced hypertrophy did not necessitate c-Myc, which indicates that unlike in cardiac muscle, where c-Myc is necessary, c-Myc is dispensable for adult skeletal hypertrophy.

Due to the possibility that genetic deletion of c-Myc was compensated by other factors during post-natal development, we further investigated the function of c-Myc

in vitro cell culture system (C₂C₁₂ myotube and myoblast). Mature myotube was differentiated and fused from single nucleated proliferating myoblast. By using a c-Myc inhibitor, Myra A, to prevent c-Myc interaction with DNA, mature Myotube did not show any defect on protein or RNA accumulation after 48h of drug administration. Consistent with the response of rRNA to overload in c-Myc conditional knock out mice, rDNA transcription and gene expression level of c-Myc were not affected either. These results reinforced the conclusion that the function of c-Myc is dispensable for post-mitotic skeletal muscle hypertrophy.

However, in proliferating cells, c-Myc plays a different role in ribosome biogenesis. It has been shown that c-Myc is essential for embryonic development. Low level of c-Myc during embryogenesis results in dwarfism due to reduced cell number rather than cell size. In myogenic cell line, c-Myc inhibits myogenic differentiation, which suggests a potential cell-stage-specific c-Myc function. To prove the different roles of c-Myc in proliferating cells and post-mitotic cells, we carried out experiments in an undifferentiated myogenic context using C₂C₁₂ myoblasts, where c-Myc was either ablated by siRNA or functionally blocked by inhibitors. Silencing of c-Myc expression with siRNA reduced cellular proliferation rates and reduced rDNA transcription. Compared to the result from silencing a well-established Pol I transcription factor RRN3, it is highly possible that the reduced proliferation rate is due to the effect of lack of c-Myc on regulation of rRNA synthesis. By using Myra-A in myoblast, reduced ribosome biogenesis and cell growth were further confirmed due to the lack of c-Myc function.

In summary, this paper suggested a cell stage specific role of c-Myc in ribosome biogenesis, which is essential for proliferating cells but not for post-mitotic cells.

4.4 PAPER IV

mTOR is essential for *de novo* rRNA synthesis. Pol I dependent 45S rDNA transcription is believed to be the rate limiting step of ribosome biogenesis. It has been shown that rDNA transcription is an early event during skeletal muscle hypertrophy. This study focused on the effect of mTOR on rDNA transcription and the importance of rDNA transcription during muscle hypertrophy.

High serum stimulation of mature C₂C₁₂ myotube resulted in increased protein content, RNA content and thicker myotubes. Consistent with previous publications, rRNA content is proportional correlated with protein synthesis rate, as rDNA transcription has an early response to high serum (Millward et al. 1973). Increased phosphorylations of mTOR/S6K1/rpS6 were observed after serum stimulation, which further confirmed the involvement of mTOR pathway during skeletal muscle

hypertrophy. To further investigate the mechanism, we used biochemical inhibitors to target different signaling molecules along the mTOR pathway, respectively, during high serum stimulation. We found that dysfunction of PI3K or mTOR but not S6K1 prevented the transcription of rDNA. S6K1 RNA interfering experiment showed similar results, which suggested that S6K1 inhibition do not affect transcription. One explanation is that S6K2 may compensate the absence of S6K1, as it has been indicated in the other cell types (McMullen et al. 2004).

Inhibition of mTOR suppressed protein synthesis rates and rDNA transcription rate. To distinguish these two processes and their regulatory effect on rDNA transcription, we applied CHX and DRB to block protein synthesis and Pol II transcription respectively. CHX showed a strong effect on suppressing rDNA transcription, which possibly due to the depletion of essential PIC factors. It also indicates that the activity of Pol I transcription requires a protein synthesis rate that above the resting level. DRB treatment showed a clear evidence that Pol II transcription was prevented during hypertrophy, which produced an imbalance between 45S pre-rRNA ETS and ITS. These data suggest an important role of Pol II transcription in Pol I elongation more than initiation. The expression of PIC factors showed different patterns between CHX and DRB, which indicates the function of mTOR signaling on protein synthesis may be distinct from its regulatory function to Pol II transcription. All these results suggest that mTOR signaling probably indirectly controls Pol I transcription by regulating Pol II mediated gene transcription.

To further understand the role of mTOR in directly regulation of Pol I transcription, we applied CX-5461, a selective Pol I inhibitor, in serum-stimulated myotubes and compared with Rapamycin. CX-5461 prevented the elevation of rDNA transcription to a similar level compared with rapamycin and inhibited hypertrophy. Furthermore, CX-5461 did not negatively affect Pol II genes. Interestingly, blockage of Pol I by CX-5461 also prevented the increase in protein synthesis, which suggests a regulatory function of Pol I transcription on protein regulation. These results indicate a necessity of *de novo* rRNA synthesis for skeletal muscle hypertrophy. In addition, we observed increased association of mTOR with the rDNA promoter following serum stimulation in a Rapamycin sensitive matter. However, Rapamycin did not affect the nuclear exclusion of mTOR, which suggests that there is a nuclear fraction of the mTOR pool regardless of its DNA binding activity at the rDNA promoter. Therefore, nuclear mTOR signaling might provide a mechanism for regulation of gene expression and Pol I transcription independent of cytoplasmic mTOR.

In conclusion, we confirmed previous findings that mTOR signaling plays an important and coordinating role in protein synthesis and ribosome biogenesis. Our

data also indicates that during muscle hypertrophy, an increase in Pol I-dependent rDNA transcription is necessary for protein synthesis, and enhanced ribosome production.

5 CONCLUSIONS

Acute resistance exercise training stimulates Pol I transcription. Gene expression following training is highly sensitive to the training state, and does not reflect the actual adaptive processes. (Paper I)

Anabolic capacity as ribosome biogenesis in muscle cells is attenuated by hypercapnia due to regulation of MuRF1 expression and muscle catabolism. (Paper II)

c-Myc gene expression is induced following mechanical loading in mouse and human. However, c-Myc is dispensable for post-natal skeletal muscle development, work-induced skeletal muscle hypertrophy and Pol I/II/III transcription. (Paper III)

Dysfunction of c-Myc in mitotic cells impairs Pol I transcription and reduces cell proliferation compared to the dispensable role of c-Myc in post-mitotic cells during C₂C₁₂ myotube hypertrophy. (Paper III)

Pol I dependent rDNA transcription is necessary for protein synthesis and C₂C₁₂ myotube hypertrophy. (Paper IV)

The regulation of mTOR on Pol I transcription does not depend on p70S6K1 function or abundance but involves mTOR-rDNAp binding in a serum- and rapamycin-dependant manner. (Paper IV)

Pol II inhibition decreases rDNA transcription, likely in part via inhibition of factors needed for RNA pol I transcription. (Paper IV)

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